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SUMMARY

Additional field samples were received and processed to supplement the supply of standard material. The new samples C^3 and D^3, after processing, were tested at the same concentration as the previous standards C^2 and D^2. The new standards contained essentially the same amount of antigenic activity as the former samples.

Several cellulose ion exchangers, and Dow ion exchange resins were evaluated for their selectivity in removing the antigenic components from field sample D^2. None of these was satisfactory.

Activated carbon was investigated again on the basis that phenol will remove the activity of sample C^2 from an aqueous solution and, therefore, it might possibly desorb the antigens from carbon. No desorption of sample C^2 antigens from C-190, 30 mesh was accomplished with a 3% phenol solution.

The feasibility of using ion exchange resin columns in series was investigated. The aim of this method was to increase the efficiency of the borate form of CG-400 resin in river water by first removing interfering substances. Two experiments have been completed. In the first one, sample C^2 was diluted 1:100 with Little Miami River water, and in the second experiment one milliliter of sample C^2 was diluted with three liters of river water. At a 1:100 dilution good recovery was obtained, while at a dilution of 1:3000 the over-all recovery was fairly poor. Although the yield was not great, this method would offer a means of detection. With an unlimited amount of sample, such as would be encountered in a river, the success of this method would depend upon the performance of the mixed bed exchanger.

All the previous work has centered on methods of recovering antigenic materials from water. During this period some time was devoted to the recovery of these materials from soil. This line of investigation seems

profitable because of the natural ion exchange properties of most soils and also the advantage of transporting soil samples.

A sample of soil was received from an area known to have been exposed to B. suis and P. tularensis. This sample was marked "Soil Sample #6." Of the various elution procedures tried, soaking with distilled water at pH 2.5 was the most successful. It would be desirable to obtain a control soil sample from the same vicinity as the actual sample, but known not to have been exposed to the test organisms.

FUTURE INVESTIGATION

Continue investigation of soil samples so one can get a correlation between activity recovered and such factors as rainfall and time of application before sampling.

Evaluate the newer ion exchange crystals such as zirconium tungstate, zirconium molybdate, and ammonium molybdophosphate. Molybdic and tungstic acids will form complexes with certain sugars in the same manner as does boric acid.

Further test the application of ion exchange resin in series on river water.

EXPERIMENTAL

I. General

The work performed during this report period covered four facets. Additional reference samples were processed, several adsorbents were screened, direct extraction with meta cresol was tried, and various elutions were made on a known positive soil sample.

II. Standard Reference Samples

Additional samples of C and D were received February 20, 1961 to supplement the supply of standard materials. Both these samples had been steam-autoclaved at 250°F for one hour before shipment. Sample C^3 was reported to have a final count of 24.4×10^9 cells/ml. and sample D to have a final count of 35×10^9 cells/ml.

The samples were clarified by passing them first through a high speed continuous Servall centrifuge and then through a Millipore membrane. The clear filtrates after being vacuum concentrated were dialyzed against distilled water for 72 hours with frequent changes of water. The dialyzates were then further concentrated to give a concentration factor of 13.3. Approximately one-half of each of the concentrates was lyophilized in 1 ml. portions. The remaining one-half of each was divided in 1 ml. portions and stored in a deep freezer.

The new reference samples were compared with the previous standard samples as to antigen content. The results of the precipitin test, which are listed in Table 1, show that the new samples have essentially the same antigen content as the previous ones.

III. Sorption Studies

A. Cellulose Ion Exchangers

The preceding report contained the results from the testing of field sample C^2 with four cellulose exchangers obtained from Nutritional Biochemicals Corporation. These exchangers had the following description:

Name	Functional Group
1. Celu ion C	Carboxymethyl
2. Celu ion D	Diethylamine
3. Celu ion P	Phosphoric acid
4. Celu ion T	Tertiary amino

The exchangers were tested with field sample D^2 by the batch method using three grams of the cellulose compound and a 1:100 dilution of D^2 in distilled water. The slurry was stirred for one hour, filtered, and the filtrate tested with homologous antiserum after being adjusted to pH 7.0.

As can be seen from the results in Table 2, none of the exchangers removed any detectable amount of activity.

B. Ion Exchange Resins

Several ion exchange resins were screened for their ability to remove the activity from field sample D^2. Five grams of each resin was placed in a 25 ml. burette, making a resin bed of approximately 10 x 70 mm. The resins were first washed with distilled water and then a solution of field sample D^2, diluted 1:100 with distilled water, was passed through the column at a flow rate of 1 ml/minute. The effluents were adjusted to pH 7.0 and tested with homologous antiserum.

The results given in Table 3 show that none of the resin removed any detectable amount of activity. Many of the resins removed all the brown color from the solution. These resins would offer a way of purifying the antigens.

C. Activated Carbon

The non-reversible sorption of field sample antigens on carbon has been reported previously. This adsorbent was investigated again on the basis that phenol removed the activity of Sample C^2 from an aqueous solution and, therefore, might possibly remove the activity from carbon.

A solution of C^2 diluted 1:100 with distilled water was passed at a flow rate of 1 ml/minute over 5 g of C-190, 30 mesh. The carbon bed was 18 x 80 mm. The column was then washed with distilled water, eluted with 100 ml. of 3% phenol solution, and the eluate dialyzed. Both the eluate and effluent were tested with homologous antiserum.

Reference to Table 4 shows that about 50% of the activity was removed under these conditions, but no desorption of the antigens was accomplished.

D. Borate Form of CG-400 Ion Exchange Resin

Previously reported data showed that the borate form of CG-400 would remove the activity from field sample C^2. It was further demonstrated that the adsorption was less when the sample was diluted with river water than with distilled water.

The failure of Amberlite IR-120 and Amberlite IRA-400 to remove the antigenic material from Sample C might provide a way of removing interfering substances.

To test this possibility a mixed bed of 5 g of IR-120, H^+ and 10 g of IRA-400, OH^- were prepared. The resin bed was 20 mm. in diameter and 130 mm. in height. A solution of sample C^2 diluted 1:100 with Little Miami River water was passed through this column at a flow rate of 1 ml. per minute. The effluent from this column was then sent over a bed, 20 x 38 mm., of CG-400 in the borate form. A flow rate of 1 ml. per minute was maintained. The borate column was then washed with 50 ml. of distilled water and eluted with 100 ml. of an 8% sodium chloride solution. After the eluate was dialyzed against distilled water, the volume was adjusted to 100 ml. The two effluents and the eluate were tested with homologous antiserum.

Inspection of Table 5 shows that the effluent from the mixed bed contained essentially the same activity as the influent. The negative results on the effluent from the CG-400 column shows that the active fraction was retained by this column. The over-all recovery was approximately 50 per cent.

From conductivity measurements on the effluent from the mixed bed column it was found that three liters of Little Miami River water could be passed through before there was any appreciable decrease in conductance. With this fact in mind, the above experiment was repeated except that sample C^2 was diluted with three liters of Little Miami River water. The flow rate

was increased to 5 ml. per minute so that all the solution could be passed through the column in one day. The results of this test are given in Table 5. Either the increased dilution or increased flow rate prevent the borate column from retaining 15% of the activity. The over-all recovery was poor, but yet the activity could be detected. With unlimited amount of solution at low concentration, which one would encounter in a river, the success of this method would depend upon the performance of the mixed bed exchanger. It is possible a small bed such as was used could remove interfering material and yet pass inorganic ions. This will be determined.

IV. Direct Extraction of Antigens

In the preceding report it was shown that the antigenic material in field sample C could be extracted from an aqueous solution by phenol. In order to make this procedure practical a less soluble compound must be used; therefore, meta cresol was tried.

Sample C^2 diluted 1:100 with distilled water was extracted twice with 5 ml. of meta cresol. The cresol layers were combined, dialyzed and the volume adjusted to 50 ml. The water layer was dialyzed to remove the cresol and then concentrated to 100 ml. A control consisting of distilled water was treated in the same manner as the sample.

The results of the precipitin test on the various layers is given in Table 6. Judging from the negative results obtained with the cresol, the phenol being so water-soluble might salt the activity out of the solution rather than being a true extraction.

V. Soil Sample

Previous work has been centered on methods of recovering antigenic material from water. During this report period a preliminary amount of work was devoted to the recovery of these materials from soil samples.

This line of investigation was followed because of the known natural ion-exchange properties of most soils and the advantage of transporting of soil samples.

A sample of soil was obtained from an area known to have been exposed to B. suis and P. tularensis. This sample was received labeled "Soil Sample #6."

Approximately 100-gram aliquots of the sample were prepared for assay by three different methods:

1) Soaking in distilled water for 24 hours (Merthiolate added).
2) Soaking in 50% aqueous phenol (w/w) for 24 hours.
3) Soaking in distilled water maintained at pH 2.5 for 24 hours.

Each of the above procedures were carried out at room temperature. At the end of the soaking period the slurry was filtered and dialyzed. After dialysis the pH was adjusted to 7.0 and the dialyzate vacuum concentrated to 5 ml. After passage through a Millipore filter, the clear filtrate was used as antigens in the tube precipitin test.

Inspection of Table 7 reveals the following results for "Soil Sample #6."

1) Soaking in distilled water does not recover the antigenic materials.
2) Soaking in 50% aqueous phenol leaches materials which give non-specific reaction with all the antisera used.
3) Soaking in distilled water maintained at pH 2.5 liberated material precipitating only with the P. tularensis antiserum.

The liberation of the active material by dilute acid indicates that the material present in the soil may be insoluble complexes or adsorbed on particles which dissolve in dilute acid, such as $CaCO_3$. The extraction of non-specific material by aqueous phenol should be investigated further.

Five-gram samples of this soil were also assayed by the above procedures and found to give negative results in all cases.

It would be desirable to obtain a control sample from the same vicinity as the actual sample from an area known not to have been exposed to B. suis or P. tularensis.

The feasibility of recovering specific antigenic material from soils would depend upon many variables such as:

1) The frequency of application to the soil.
2) Percolation of soil.
3) Rainfall.
4) Physical properties of soil.
5) Chemical properties of soil.
6) Microbiological activity of soil.
7) Temperature.
8) Time after application before sampling.

TABLE 1

COMPARISON OF REFERENCE SAMPLES

Antiserum	Sample	Dilution of Sample (Conc. Factor 13.3) 1:256	1:512	1:1024	1:2048
# 43	C^2	3	3	1	0
# 43	C^3	4	4	2	1

Controls

C^2 1:256 + saline = neg.
C^3 1:256 + saline = neg.

antiserum # 43 + saline = neg.

Antiserum	Sample	Dilution of Sample (Conc. Factor 13.3) 1:256	1:512	1:1024	1:2048
# 45	D^2	5	5	3	2
# 45	D^3	5	5	3	2

Controls

D^2 1:256 + saline = neg.
D^3 1:256 + saline = neg.

antiserum # 45 + saline = neg.

TABLE 2

CELLULOSE EXCHANGERS WITH FIELD SAMPLE D

	Dilution of Filtrates			
	As is	1:2	1:4	1:8
Standard D^2 - 1:100	5	5	4	3
Celu Ion C	5	5	4	3
Celu Ion D	5	5	4	3
Celu Ion P	5	5	4	3
Celu Ion T	5	5	4	3

Controls
Filtrates + saline = neg.
Antiserum # 44 + saline = neg.

TABLE 3

ION EXCHANGE RESIN STUDIES

RESIN	Dilution of Effluents As is	1:2	1:4	1:8
None - Standard D^2 1:100	4	3	2	1
Dowex 1 X 2, Cl^-, 50 - 100 mesh	4	3	2	1
Dowex 1 X 8, Cl^-, 50 - 100 mesh	4	3	2	1
Dowex 2 X 4, Cl^-, 20 - 50 mesh	4	3	2	1
Dowex 2 X 8, Cl^-, 50 - 100 mesh	4	3	2	1
Dowex 21K, Cl^-, 50 - 100 mesh	4	3	2	1
Dowex 50W X 1 H^+ 50 - 100 mesh	4	3	2	1
Dowex 3, free base, 20 - 50 mesh	4	3	2	1
Bio-Rex 63, Na^+, 50 - 100 mesh	4	3	2	1

Controls
Effluents + saline = neg.
Antiserum # 37 + saline = neg.

TABLE 4

SORPTION OF C^2 ANTIGENS WITH C-190 CARBON

SAMPLE	Dilution of Sample As is	1:2	1:4	1:8
C^2 (diluted 1:100)	4	3	2	1
Carbon effluent	2	1	0	0
3% Phenol eluate	0	0	0	0

Controls
C^2 Standard + saline = neg.
Effluent + saline = neg.
Eluate + saline = neg.
Antiserum # 33 + saline = neg.

TABLE 5

ION EXCHANGE COLUMNS IN SERIES

Sample C^2 in L.M.R. 1:100

SAMPLE	Dilution As is	1:2	1:4	1:8
C^2 Standard - 1:100	4	3	2	1
Effluent mixed bed, 100 ml.	4	3	2	1
Effluent CG-400, 100 ml.	0	0	0	0
Eluate CG-400, 100 ml.	3	1	0	0

Controls - neg.

Sample C in L.M.R. 1:3000

SAMPLE	Dilution As is	1:2	1:4	1:8
C^2 Standard 1:100	5	5	4	2
Effluent CG-400, 50 ml.	2	1	0	0
Eluate CG-400, 25 ml.	2	1	0	0

Controls = neg.

TABLE 6

DIRECT EXTRACTION OF C^2 WITH CRESOL

SAMPLE	Dilution As is	1:2	1:4	1:8
Aqueous layer - 100 ml.	3	2	1	0
Phenol layer - 50 ml.	0	0	0	0
Aqueous control	0	0	0	0
Standard C^2 - 1:100	4	3	2	1

Controls
Antiserum # 33 + saline = neg.
Standard C^2 + saline = neg.

TABLE 7

EXTRACTION OF SOIL SAMPLE #6

Antiserum	Distilled Water Extract			
	As is	1:2	1:4	1:8
B. suis	0	0	0	0
P. tularensis	0	0	0	0
P. pestis	0	0	0	0

Antiserum	50% Aqueous Phenol Extract			
	As is	1:2	1:4	1:8
B. suis	2	1	0	0
P. tularensis	2	1	0	0
P. pestis	2	1	0	0
Casein	2	2	1	0
Egg albumin	2	1	0	0
Monkey anti-rabbit	2	1	0	0
Normal rabbit	2	1	0	0

Antiserum	Distilled Water pH 2.5 Extract			
	As is	1:2	1:4	1:8
B. suis	0	0	0	0
P. tularensis	4	3	2	1
P. pestis	0	0	0	0
Monkey anti-rabbit	0	0	0	0

Proper controls were included